Immunology 1985 **54** 163

Inhibition of human neutrophil receptor-mediated uptake of N-formyl-met-leu-phe by platelet factor 4 (59–70)

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Summary. Human platelet factor 4 (PF4) and a substituent dodecapeptide designated PF4(59–70) elicited human neutrophil and monocyte chemotaxis with a similar concentration-dependence and maximal responses equal to that attained by chemotactic fragments of C5 (C5fr). At maximally chemotactic concentrations, PF4(59–70) stimulated the secretion by neutrophils of approximately 40% and 60% of the respective quantities of β-glucuronidase and β-glucosaminidase released by 10^{-6} M N-formyl-methionyl-leucyl-phenylalanine (fMLP). In contrast to the deactivation of chemotaxis achieved by preincubation of neutrophils with other chemotactic factors, prior exposure to 10^{-6}M PF4(59–70) for 2 min, or 20 min at 37°, enhanced by 1·5- to 2-fold the chemotactic responses of neutrophils evoked by optimal concentrations of fMLP, C5fr, leukotriene B_4, and PF4(59–70). Concentrations of PF4(59–70) which enhanced neutrophil chemotaxis inhibited the rate of receptor-mediated internalization of [^{3}H]fMLP at 37° and 18°, but at 0° failed to alter the binding affinity or the number of receptors for [^{3}H]fMLP. Preincubation of neutrophils at 37° with concentrations of PF4(59–70) which enhanced neutrophil chemotaxis also did not affect the subsequent binding of [^{3}H]fMLP at 0°. The inhibition by PF4(59–70) of the receptor-mediated internalization of [^{3}H]fMLP was not mimicked by other positively charged compounds. The specific inhibition of receptor-mediated internalization of fMLP may explain the enhanced chemotactic responsiveness of neutrophils preincubated with PF4(59–70).

Abbreviations: chemotactic fragments of C5, C5fr; Hanks' balanced salt solution, HBSS; HBSS containing 0·1 g ovalbumin per 100 ml and 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (pH 7·3), HBSS-OA; high power field, h.p.f.; N-formyl-methionyl-leucyl-phenylalanine, N-formyl-met-leu-phe or fMLP; penta-lysine, $(Lys)_5$; platelet factor 4, PF4; 5(S),12(R)-di-hydroxy-eicosa-6, 14-cis-8, 10-trans-tetraenoic acid, leukotriene B4, LTB4.

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INTRODUCTION

The exposure of human platelets to aggregating stimuli results in the release from α-granules of platelet factor 4[3] (PF4), a 70-amino acid polypeptide which binds heparin with high affinity (Deuel *et al.*, 1977; Handin & Cohen, 1976). The amino acid sequence of human PF4 revealed that the carboxy-terminal portion is hydrophobic and highly basic, likely to mediate the binding of PF4 to heparin, and may have a specific conformation in solution (Deuel *et al.*, 1977). PF4 and the carboxy-terminal substituent peptide PF4(59–70), leu-tyr-lys-lys-ile-ile-lys-lys-leu-leu-glu-ser, elicit chemotactic responses of human neutrophils and monocytes *in vitro* (Deuel *et al.*, 1981; Goldman, Hannah & Goetzl, 1982; Osterman *et al.*, 1982). The capacity of PF4 and PF4(59–70) to enhance the chemotactic responses of human neutrophils to N-formylated-peptides and some other factors was examined in relation

to the binding properties and cellular characteristics of receptors for fMLP. The data presented show that PF4 and PF4(59–70) do not alter the binding of fMLP to human neutrophil receptors, but effectively suppress the receptor-mediated internalization of fMLP by neutrophils.

MATERIALS AND METHODS

Materials

Hanks' balanced salt solution without phenol red (HBSS; M.A. Bioproducts, Bethesda, MD), phenolphthalein glucuronic acid solution, sodium dodecyl sulphate, N-formyl-met-leu-phe, 4-methylumbelliferyl-β-D-glucoside (Sigma Chemical Co., St Louis, MO.), [^{3}H]fMLP (55·6 Ci/mM; New England Nuclear, Boston, MA), ovalbumin (Miles Laboratories, Inc., Elkhart, Ind.), Ficoll-Hypaque and 6% (w/v) dextran 70 in normal saline (Pharmacia Fine Chemicals, Piscataway, NJ), *n*-butyl phthalate (Fisher Scientific Co., Medford, MA), dinonyl phthalate (ICN Pharmaceuticals Inc., Plainview, NY), penta-lysine (Lys_5, Vega Biochemicals, Tucson, AZ), hexane sulphonate (Rainin Instruments, Inc., Woburn, MA), cytochalasin B (Aldrich Chemical Co., Milwaukee, WI), and 0·4 ml polypropylene tubes (Walter Sarstedt, Inc., Princeton, NJ) were obtained from the suppliers noted. All organic solvents were distilled from glass (HPLC grade; Burdick and Jackson Laboratories, Inc., Muskegon, MI). Fragments of the fifth component of complement (C5fr) were prepared from yeast-activated human serum as previously described (Goetzl & Hoe, 1979). Synthetic 5(S), 12(R)-di-hydroxy-eicosa-6, 14-cis-8, 10-trans-tetraenoic acid (leukotriene B_4, LTB_4) was kindly supplied by Dr Rokach of Merck-Frosst Laboratories, Quebec, Canada.

Preparation of PF4 and PF4(59–70)

PF4 was extracted from human platelets by incubation of 5×10^{11} platelets for 1 hr at 4° in 100 ml of 0·15 M NaCl adjusted to pH 2·0 with 4 M HCl. The PF4 in the supernate was recovered, concentrated, and purified by heparin-sepharose affinity chromatography as described (Handin & Cohen, 1976) and stored in 2 M NaCl-0·05 M Tris-HCl (pH 8·6) at −20°. The carboxy-terminal dodecapeptide of PF4 (PF4(59–70)) was synthesized by solid-phase techniques and purified as described (Brindley, Sweet & Goetzl, 1983). The PF4(59–70) preparation yielded a single ninhydrin positive spot after thin-layer chromatography on silica gel ($R_F = 0·14$) and cellulose ($R_F = 0·83$) developed with H_2O/*n*-butanol/ethyl acetate/acetic acid (1:1:1:1, v/v). A single peak of OD_{210} with a retention time of 26·9 min was detected by high performance liquid chromatography on a 5 μm octadecylsilane reverse-phase column (4·6 mm × 250 mm, Altex-Beckman Scientific, Inc., Berkeley, CA), equilibrated in 20% acetonitrile: water (v/v) containing 10 mM hexane sulphonate and 10 mM H_3PO_4 (pH 2·9) and developed with a 40 min gradient beginning at the time of sample injection and increasing to 80% (v/v) acetonitrile: water with the same additives.

Preparation of human neutrophils and mononuclear leucocytes

Human neutrophils and mononuclear leucocytes were prepared as described (Geotzl & Hoe, 1979) from sodium citrate-anticoagulated venous blood of normal donors. Erythrocytes were removed by dextran sedimentation followed by hypotonic lysis with distilled water at 4° for 20 sec before isotonicity was restored by adding 0·6 M KCl. Neutrophils of 96% or greater purity and mononuclear leucocytes composed of 13–24% monocytes (range, $n = 4$) were obtained by centrifugation of mixed leucocytes on Ficoll-Hypaque cushions (Boyum, 1968). The purified leucocytes were washed and resuspended in HBSS containing 0·1 g ovalbumin per 100 ml and 10 mM N-2-hydroxy-ethyl-piperazine-N′-2-ethanesulphonic acid (pH 7·3) (HBSS-OA), stored at 4° and used within 2 hr.

Chemotaxis and enzyme release

Chemotactic migration was performed in modified Boyden chambers (Adaps Inc., Dedham, MA) assembled with micropore filters of 3 μm pore diameter (Sartorius, Göttingen, West Germany) for assessing the migration of neutrophils or with micropore filters of 8 μm pore diameter for assessing the migration of monocytes as previously described (Brindley *et al.*, 1983; Goetzl *et al.*, 1980; Goetzl, 1980). Leucocytes were enumerated microscopically in 10 high power fields (h.p.f.), five from each of duplicate filters, at a depth of 80–100 μm from the cell source. The depth for counting was selected to achieve a background count of three to eight leucocytes per h.p.f. The response is expressed as net leucocytes per h.p.f., after subtraction of background migration in control chambers lacking a stimulus. The altered chemotactic migration of pretreated PMN leucocytes is denoted as a percentage of the migration of replicate portions of PMN leucocytes preincubated in HBSS-OA alone. Statistical

analyses were performed with a standard two-sample Student's *t*-test.

The stimulation of release of lysomal granule constituents was determined by incubating 2×10^6 neutrophils in 0·4 ml HBSS-OA containing the stimulus and 5μg/ml of cytochalasin B at 37° for 30 min. The supernatant was assayed colorimetrically for β-glucuronidase (Goetzl & Pickett, 1980) and fluorometrically for β-glucosaminidase (Leaback & Walker, 1961) as previously described. The amount of release of each enzyme is expressed as a percentage of the total amounts of activity present in the replicate suspensions of neutrophils that had been disrupted by sonication on ice.

Measurement of the binding of [^{3}H]fMLP to human neutrophils

Ethanol solutions of [^{3}H]fMLP containing 0·2% mercaptoethanol were reduced to dryness with a stream of nitrogen and resuspended in HBSS-OA (pH 6·8). Solid fMLP was dissolved initially in dimethyl sulphoxide and diluted in sufficient HBSS to give a final concentration of 1mM fMLP with 0·5% DMSO (v/v). This stock solution was stored under N_2 at 4° and made fresh every two weeks.

The binding of [^{3}H]fMLP to neutrophils was determined by incubating 5×10^6 neutrophils with [^{3}H]fMLP in the presence or absence of other ligands in a final volume of 250 μl HBSS-OA (pH 6·8). Standard incubation conditions were 30 min at 0°, 120 min at 18°, and 60 min at 37°. After the incubation period, each suspension was layered on 0·1 ml of a mixture of *n*-butyl phthalate and dinonyl phthalate (7:2, v/v) in a 0·4 ml polypropylene tube and centrifuged for 30 sec at 8000 ***g*** in a Beckman microfuge B (Beckman Instruments Inc., San Jose, CA). The tip of the polypropylene tube containing the neutrophil pellet was cut off with a razor blade, and the contents of the tube were resuspended with a Pasteur pipette in 1 ml of 0·1 g% sodium dodecyl sulphate to which was added 10 ml of Hydrofluor (National Diagnostics Inc., Somerville, NJ). The amount of radioactivity in the pellet was quantified in a β-scintillation counter (Tracor Analytical Inc., Mark III, Des Plaines, IL.). The amount of free radioactivity in each solution was determined by similarly quantifying the radioactivity in a 50 μl aliquot of the aqueous layer on top of the phthalate oil layer.

The total number of moles of [^{3}H] fMLP bound to the neutrophils was determined by dividing the c.p.m. bound to the cell pellet by the specific activity of the [^{3}H]fMLP and the counting efficiency. The amount of radioactivity bound in the presence of 1×10^{-5}M non-radioactive fMLP was divided by the same values for the specific activity and counting efficiency to determine the non-specific binding component. The number of moles specifically bound to the neutrophils was derived by subtracting the non-specific binding from the total binding. The concentration of [^{3}H]fMLP free in solution was calculated using the amount of radioactivity present in 50 μl of the aqueous phase after separation of cells and supernatant.

RESULTS

PF4 and PF4(59–70) elicited human neutrophil chemotactic responses at concentrations of 10^{-7} M–3×10^{-5}M (Fig. 1a). The peptide concentration-response relationship was similar for both factors and the magnitude of the responses to 3×10^{-6}M PF4 and 10^{-5}M PF4(59–70) was equal to that elicited by an optimal concentration of C5fr. PF4(59–70) stimulated human monocyte chemotaxis with a concentration-dependence similar to that observed for neutrophil chemotaxis (Fig. 1b) and achieved a response at 3×10^{-5}M which was equal to the response elicited by an optimal concentration of C5fr. A modified checkerboard assay established that PF4 and PF4(59–70) elicit chemotactic responses of both neutrophils and monocytes and that an increase in random migration alone cannot account for the observed increase in migration of either type of leucocyte. PF4 at a concentration of 10^{-6}M on the stimulus side alone and both sides of the filter, respectively, elicited responses of $28{\cdot}2 \pm 4{\cdot}1$ and $8{\cdot}8 \pm 1{\cdot}4$ neutrophils/h.p.f. (mean $\pm$ SD, $n=4$) and $31{\cdot}1 \pm 3{\cdot}5$ and $8{\cdot}3 \pm 3{\cdot}2$ monocytes/h.p.f., while 10^{-5} M PF4(59–70) on the stimulus side alone and both sides elicited responses of $30{\cdot}4 \pm 3{\cdot}2$ and $9{\cdot}9 \pm 1{\cdot}6$ neutrophils/h.p.f., and $33{\cdot}2 \pm 3{\cdot}0$ and $8{\cdot}6 \pm 1{\cdot}4$ monocytes/h.p.f. PF4(59–70) stimulated the release of lysosomal enzymes from human neutrophils only minimally in the presence of cytochalasin B. The amount of β-glucuronidase and β-glucosaminidase released by neutrophils in response to PF4(59–70) was maximal at 10^{-4}M, where the respective levels reached $12{\cdot}2 \pm 7{\cdot}6\%$ (mean $\pm$ SD, $n=8$) and $23{\cdot}5 \pm 10\%$ ($n=4$) respectively. These values are significantly lower than the release of $30{\cdot}4 \pm 14\%$ of β-glucuronidase ($n=8$, $P=0{\cdot}002$ relative to 10^{-4}M PF4) and of $39{\cdot}6 \pm 19{\cdot}7\%$ of β-glucosaminidase ($n=3$, $P=0{\cdot}05$), which were achieved concurrently by 10^{-6}M

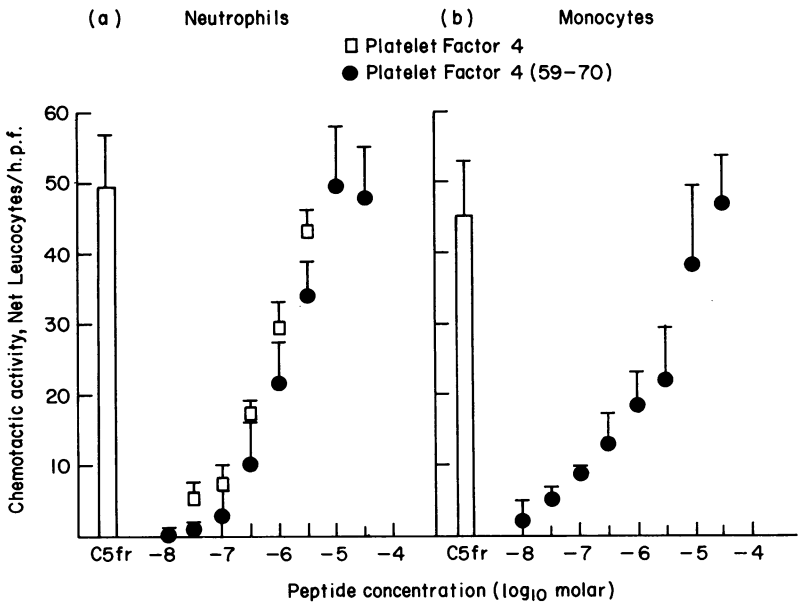

Figure 1. Stimulation of human leucocyte chemotaxis by (□) PF4 and (●) PF4(59–70). Each point and bracket represents the mean ± SD of four experiments with leucocytes from different normal subjects. The bars depict the chemotactic response elicited by a maximal dose of C5fr.

fMLP. In the absence of cytochalasin B, no net release of lysosomal enzymes was observed with PF4(59–70).

The pretreatment of neutrophils with chemotactic concentrations of PF4(59–70) at 37° enhanced by up to 2·5-fold the susequent chemotactic responses of the neutrophils to both PF4(59–70) and other chemotactic stimuli (Table 1). The enhancement of chemotactic responses was greatest after 2 min of pretreatment with 10^{-6}M PF4(59–70). In contrast to the enhancement observed with PF4(59–70), pretreatment with fMLP significantly reduced the subsequent chemotactic migration of the neutrophils to fMLP and PF4(59–70), but not to C5fr or leukotriene B_4.

The effects of PF4(59–70) on human neutrophil receptors for fMLP were examined in order to determine the basis for the stimulation of chemotactic responses. At 0°, a concentration of PF4(59–70) which stimulated chemotaxis optimally failed to alter significantly either the dissociation constant (K_d) for the binding of [^{3}H]fMLP to neutrophils or the maximal number of binding sites for fMLP per neutrophil (Fig. 2a, b). The specific binding of fMLP to neutrophils from four experiments, analysed according to the method of Scatchard (1949), revealed a K_d (mean ± SD) of 26·2 ± 3·6 × 10^{-9}M and 17,300 ± 5800 sites per neutrophil in the absence of PF4(59–70), and a K_d of 26·8 ± 2·4 × 10^{-9} M and 17,400 ± 7600 sites per neutrophil in the presence of PF4(59–70). Thus, PF4(59–70) does not compete with [^{3}H]fMLP for binding to the fMLP receptor at 0°. The fMLP specifically bound to neutrophils at 0° is at equilibrium with the extracellular fluid phase since the binding is rapid, reaching a stable plateau level within 10 min (Fig. 3), and is reversible. Within 40 min after the addition of 10 μM non-radioactive fMLP, 84·2 ± 7·0% (mean ± SD, $n = 4$) of the [^{3}H]fMLP specifically bound at 0° has been displaced (Fig. 4). At higher temperatures, 18° and 37°, the amount of [^{3}H]fMLP specifically bound to the neutrophils increases steadily for longer periods of time than at 0° (Fig. 3). Virtually none of the [^{3}H]fMLP specifically bound to neutrophils at 18° was displaced by incubation with 10 μM fMLP at 0° for 60 min (Fig. 4). Of the [^{3}H]fMLP specifically bound to neturophils at 37°, only 16 ± 8% (mean ± SD, $n = 4$) was displaced by 10 μM fMLP after 60 min at 0°. The non-displaceable binding appears to represent [^{3}H]fMLP which has been internalized subsequent to binding to the receptor (Zigmond, Sullivan & Lauffenberger, 1982; Vitkauskas, Showell & Becker, 1980). The specific, non-displaceable binding of

Table 1. Effect of pretreatment with fMLP or PF4(59–70) on the chemotactic responses of human neutrophils

Chemotactic* stimulus	Pretreatment conditions			
	10^{-7}M fMLP		10^{-6}M PF4(59–70)	
	2 min	20 min	2 min	20min
10^{-6}M PF4(59–70) ($n=4$)	58±26†	53±41‡	210±69†	163±71‡
3×10^{-6}M PF4(59–70) ($n=4$)	70±50	61±42‡	230±86‡	194±70‡
10^{-7}M fMLP ($n=3$)	35±7‡	44±25†	223±77‡	123±25
10^{-6}M fMLP ($n=4$)	37±19‡	27±5‡	169±39†	139±21‡
C5fr ($n=3$)	88±10	95±9	202±36†	138±40
Leukotriene B_4 ($n=3$)	76±17	90±11	249±46‡	211±49‡

* The data is expressed as the percentage of the control response (100%), mean±SD. The 100% value represents the chemotactic response of neutrophils pretreated with buffer alone for 20 min at 37°. The 100% value (mean±SD) for each stimulus was 10^{-6}M PF4(59–70)=7·7±2·0; 3×10^{-6} PF4(59–70)=13·5±2·1; 10^{-7}M fMLP=16·2±2·5; 10^{-6} fMLP=28·1±8·9; C5fr=23·7±9·8; leukotriene B_4=17·9±3·2.

† $P<0.01$ (difference between the responses of neutrophils preincubated with a peptide, or with buffer alone).

‡ $P<0.05$.

[^{3}H]fMLP to neutrophils at 18° and 37° was markedly reduced by 4×10^{-5}M PF4(59–70) (Fig. 3). The decrease in the total amount of [^{3}H]fMLP bound to neutrophils was significant after 20 min at 37° ($n=4$, $P=0.01$), but not 60 min at 18° ($n=3$, $P=0.08$).

The receptor-mediated internalization of [^{3}H]fMLP at 18° was inhibited by PF4(59–70) in a concentration-dependent manner, with half-maximal inhibition at 1×10^{-6}M PF4(59–70) (Fig. 5), and accounted for the bulk of the total inhibition at 3×10^{-7}M–3×10^{-6}M PF4(59–70). Concentrations of PF4(59–70) of 10^{-5}M or greater also inhibited the non-receptor-mediated internalization of [^{3}H]fMLP, but the major effect of even the highest concentrations of PF4(59.70) was the inhibition of receptor-mediated internalization. When receptor-mediated internalization of different concentrations of [^{3}H]fMLP (1×10^{-8}M–3×10^{-7}M) was determined after 2 hr at 18° in the presence and absence of PF4(59–70), PF4(59–70) was markedly inhibitory at all of the concentrations of fMLP. A double reciprocal plot of the data suggested that the inhibition of neutrophil internalization of [^{3}H]fMLP by PF4(59–70) was non-competitive. The effect of the pentapeptide $(Lys)_5$ and of NH_4Cl on the internalization of [^{3}H]fMLP by neutrophils was determined to see if other positively charged reagents could mimic the inhibitory effects of PF4(59–70). 10^{-5}M PF4(59–70), 10^{-3}M $(Lys)_5$, and 10^{-2}M NH_4Cl produced a mean decrease of 25%, 4% and 2%, respectively, in the amount of [^{3}H]fMLP internalized by neutrophils in 2 hr at 18°.

In order to assess the possibility the PF4(59–70) may also alter fMLP receptor expression in the absence of fMLP, neutrophils were pretreated with PF4(59–70) at 37° for 30 min and the binding of [^{3}H]fMLP then was quantified at 0° (Table 2). A significant increase in the specific binding of [^{3}H]fMLP was observed at concentrations of PF4(59–70) greater than 10^{-5}M. However, as pretreatment of neutrophils with 10^{-6}M PF4(59–70) produced no significant increase in the subsequent binding of [^{3}H]fMLP, it is unlikely that an increase in the number or affinity of

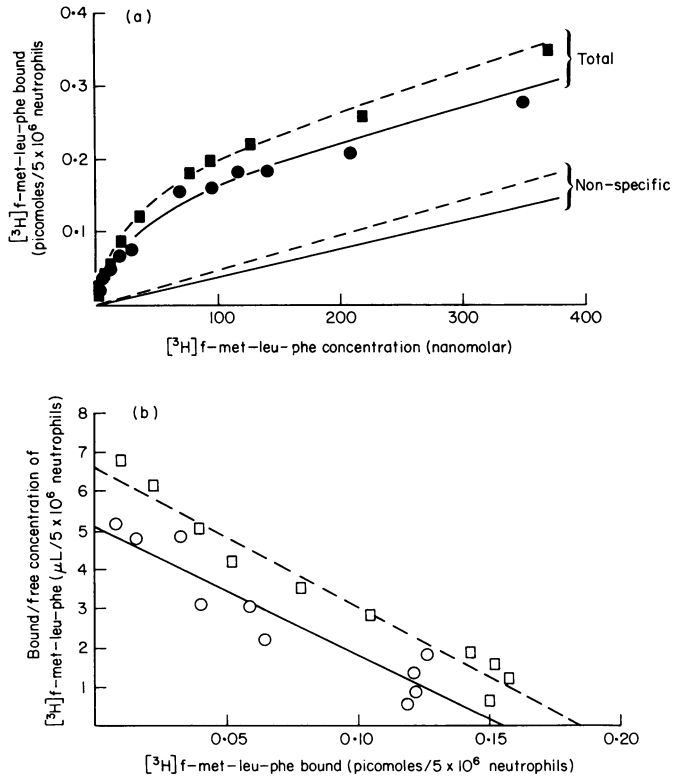

Figure 2. The effect of PF4(59–70) on the binding of [^{3}H]fMLP to human neutrophils at 0°. Neutrophils, 5×10^6, were incubated for 15 min with the indicated concentrations of [^{3}H]fMLP at 0° in (●, ○) the absence and (■, □) the presence of 4×10^{-5}M PF4(59–70). Each data point is the mean of duplicate determinations. (a), total binding and non-specific binding of [^{3}H]fMLP in (●——●) the absence and (■– – –■) the presence of 4×10^{-5}M PF4(59–70). The percentage of the total [^{3}H]fMLP bound to the neutrophils in the presence of 10^{-5}M fMLP was used to calculate the non-specific binding parameter (NS) for [^{3}H)fMLP. The total binding (TB) curves were calculated from the formula:

$$\text{Total binding} = \frac{n \times (\text{fMLP})}{(\text{fMLP}) + K_d} + \text{NS} \times (\text{fMLP})$$

where n = maximal moles of fMLP specifically bound per 5×10^6 neutrophils, (fMLP) = fMLP concentration, and K_d = dissociation constant.

(b), Scatchard plot of the specific binding data [TB – NS × (fMLP)] taken from (a). The straight lines were fit by linear regression analysis and the slope and the x-intercept were used to calculate the values of K_d and the total number of sites per neutrophil. In this representative experiment (one of four), neutrophils expressed $1{\cdot}9 \times 10^4$ sites per neutrophil with a K_d of $3{\cdot}1 \times 10^{-8}$M for fMLP and $2{\cdot}2 \times 10^4$ sites per neutrophil with a K_d of $2{\cdot}8 \times 10^{-8}$M in (○——○) the abscences, and (□——□) the presence of PF4(59–70), respectively.

receptors for fMLP could alone account for the observed enhancement of neutrophil chemotactic migration after pretreatment with PF4(59–70). In two parallel experiments, pretreatment of neutrophils with 10^{-7}M fMLP at 37° for 20 min, followed by extensive washing of the neutrophils at 0°, decreased binding of [^{3}H]fMLP at 0° by a mean of 55%. This phenomenon in neutrophils has been termed down-regulation of chemotactic receptors (Zigmond *et al.*, 1982). When 2×10^{-5}M PF4(59–70) was present during the pretreatment with 10^{-7}M fMLP, the subsequent binding of [^{3}H]fMLP at 0° was decreased by a mean of 60% in the same two experiments. Thus, PF4(59–70) appeared to have no effect on the down-regulation of fMLP

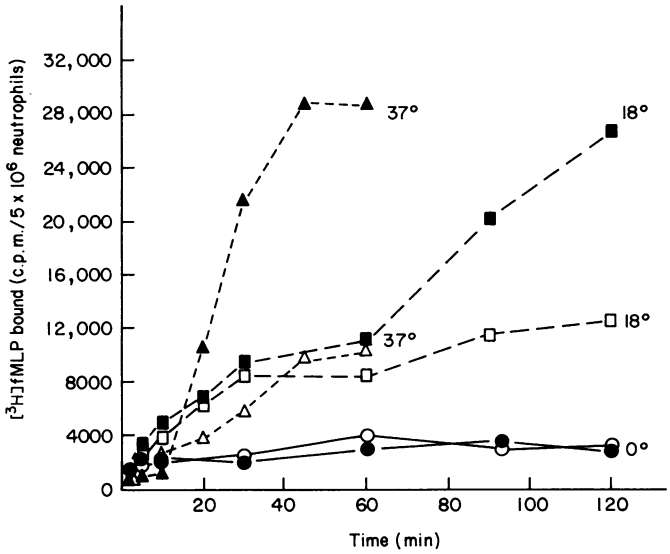

Figure 3. The effect of PF4(59–70) on the time course of binding of [^{3}H]fMLP to human neutrophils. Human neutrophils were incubated with 10^{-8}M [^{3}H]fMLP at (○, ●) 0°, (□, ■) 18° and (△, ▲) 37° in the absence (solid symbols) and the presence (open symbols) of 4×10^{-5}M PF4(59–70). Each point represents the mean of duplicate determinations.

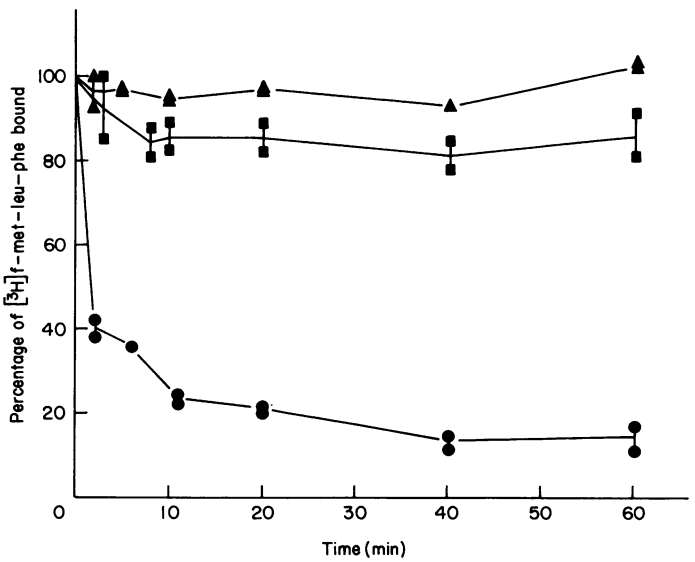

Figure 4. Time course of dissociation of [^{3}H]fMLP from human neutrophils. Neutrophils were incubated with 10^{-8}M [^{3}H]fMLP for 30 min (●) 0°, (■) 60 min at 18° and (▲) 30 min at 37°. At time 0 (100% binding), fMLP in ice-cold HBSS-OA was then added and the neutrophil suspensions were incubated on ice for the indicated times. Each data point represents a single determination.

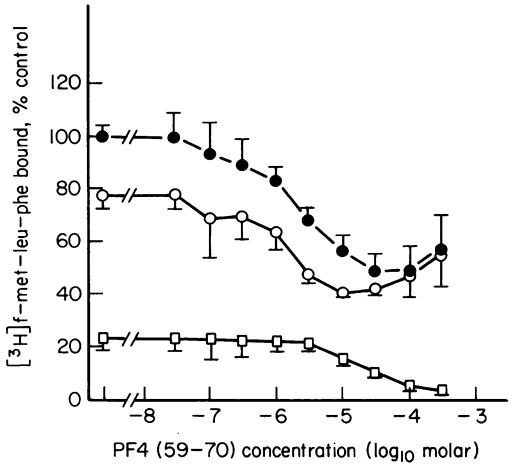

Figure 5. Concentration-dependence of the inhibition by PF4(59–70) of binding of [^{3}H]fMLP to human neutrophils. Neutrophils, 5×10^6 were incubated with 10^{-8}M [^{3}H]fMLP and different concentrations of PF4(59–70) at 18° for 2 hr. The receptor-mediated binding (○) was determined by subtracting the amount of [^{3}H]fMLP bound in the presence of 10^{-5}M fMLP (□) from the total binding (●). The total amount of [^{3}H]fMLP bound in the absence of PF4(59–70) was defined as the 100% control level of binding.

receptors induced by preincubation with unlabelled fMLP.

DISCUSSION

The synthetic dodecapeptide PF4(59–70), a substituent peptide of the 70 amino acid peptide PF4, elicits chemotactic responses from both human monocytes and neutrophils similar in magnitude to that elicited by PF4 at nearly equal concentrations, and elicits maximal chemotactic responses equal to those elicited by optimal concentrations of C5fr. A modified checkerboard assay (Goetzl & Gorman, 1978; Zigmond & Hirsch, 1973) revealed that the increased leucocyte migration elicited by PF4 and PF4(59–70) is a chemotactic response and is not solely due to an increase in random migration. The similarities in the dose-response curves for the stimulation by PF4 and PF4(59–70) of human neutrophil chemotaxis suggests that PF4(59–70) can be used to study the activation and modulation of neutrophil function by PF4. PF4(59–70) stimulated only a minimal release from neutrophils of lysosomal enzymes in the presence of cytochalasin B. Compared to an optimal concentration of fMLP, only one-third to one-half the amount of granule enzymes are released in response to an optimal concentration of PF4(59–70).

Table 2. Effect of PF4(59–70) pretreatment at 37° on the specific binding of (^{3}H)f-met-leu-phe at 0°

PF4(59–70) concentration	Binding of (^{3}H)f-met-leu-phe (mean percent of buffer control ±SD)
10^{-7}M	97·0±10·4
3×10^{-7}M	105·4±12·3
10^{-6}M	106·2±25·6
3×10^{-6}M	106·2±25·6
10^{-5}M	114·6±25·6
3×10^{-5}M	122·3±10·4*
10^{-4}M	139·0±18·7*

Neutrophils, 5×10^6 were incubated with PF4(59–70) for 30 min at 37°, centrifuged, and resuspended in 10^{-7}M (^{3}H)fMLP at 0° for 15 min. The amount of (^{3}H)fMLP bound to neutrophils pretreated with buffer alone is defined as 100%. $n = 5$ experiments.
* $P < 0.05$.

Preincubation of neutrophils with 10^{-6}M PF4(59–70) led to a 1·5- to 2-fold increase in the subsequent chemotactic response of the neutrophils elicited by PF4(59–70), fMLP, C5fr, and leukotriene B_4. This stimulatory effect of PF4(59–70) is in marked contrast to the inhibitory effect of fMLP preincubation on the subsequent chemotactic response elicited by fMLP and PF4(59–70). Earlier studies have shown that preincubation of human neutrophils with fMLP, C5fr, or leukotriene B_4 (Ward & Becker, 1968; Nelson *et al.*, 1978; Goetzl *et al.*, 1981) produces a substantial decrease in the subsequent chemotactic response elicited by the factor present during the preincubation, while the chemotactic response towards other factors remains unaffected, a phenomena termed 'stimulus-specific' deactivation. The receptor-mediated internalization of fMLP by neutrophils which occurs at 18° and 37° is initiated by the binding of fMLP to its receptor followed by the internalization and redistribution of fMLP into a non-dissociable compartment (Sullivan & Zigmond, 1980; Zigmond *et al.*, 1982). The observed decrease in the number of surface binding sites expressed for radiolabelled N-formyl peptides (Sullivan & Zigmond, 1980), in parallel with the 'stimulus-specific' deactivation of neutrophil chemo-

taxis (Vitkauskas *et al.*, 1980; Donabedian & Gallin, 1981), led us to examine whether PF4(59–70)-induced changes in the binding or processing of fMLP by the neutrophil could account for the stimulatory effects of PF4(59–70) on chemotaxis. Chemotactic concentrations of PF4(59–70) had no effect on the rate of [^{3}H]fMLP binding to human neutrophils or on the affinity or number of receptor binding sites expressed by the neutrophil for [^{3}H]fMLP at 0°. The predominant effect of PF4(59–70) is the dose-dependent inhibition of the apparently irreversible association of [^{3}H]fMLP with specific neutrophil receptors at 18° and 37°. Although the phenomenon observed might be attributable either to a conformational change in the combining site of occupied receptors, which precludes displacement of bound ligand, or to internalization of occupied receptors, published data (Zigmond *et al.*, 1982) favour the latter interpretation. The inhibition of the receptor-mediated internalization of [^{3}H]fMLP is specific for PF4(59–70) and not solely due to the highly basic character of the peptide. Neither $(Lys)_5$ nor NH_4Cl inhibited the receptor-mediated internalization of [^{3}H]fMLP at 100-fold and 1000-fold higher concentrations, respectively, compared to 10^{-5}M PF4(59–70).

The stimulation of neutrophil chemotaxis and the inhibition of receptor-mediated internalization of fMLP by chemotactic concentrations of PF4(59–70) raises the possibility that the internalization of chemotactic factors may be an important mechanism for modulating the chemotactic responsiveness of the neutrophil. For instance, the receptor-mediated internalization of fMLP could serve as a mechanism for lowering the effective concentration of fMLP at the cell surface. This role has been suggested for myeloperoxidase-dependent oxidative inactivation of fMLP, which limits the extent and duration of the amount of superoxide generated by human neutrophils (Stendahl *et al.*, 1984). Similarly, inhibition of the receptor-mediated internalization of fMLP could reduce the rate at which the extracellular fMLP concentration is lowered and, thus, enhance the chemotactic response elicited by fMLP. Alternatively, the receptor-mediated internalization of fMLP could modulate the extent of neutrophil activation, either by altering the average length of fMLP receptor occupancy, or by regulating the surface expression of the fMLP receptor.

The stimulation by PF4(59–70) of neutrophil chemotaxis elicited by C5fr and LTB_4 suggests that PF4(59–70) may also modulate the expression of the neutrophil receptors for these structurally different stimuli. Separate populations of neutrophil receptors for C5a (Chenoweth & Hugli, 1978) and LTB_4 (Goldman & Goetzl, 1984) have been identified and shown to be distinct from the receptors for N-formylated peptides, such as fMLP. Although it has not been established that the receptors for chemotactic factors other than fMLP mediate internalization or other cellular processing of the bound factors, it is possible to propose several mechanisms by which PF4(59–70) may enhance chemotactic responses without altering the characteristics of binding of the factors to their respective receptors. PF4(59–70) may bind to a regularory site which is common to all chemotactic receptors, may bind to separate PF4 receptors capable of interacting with and affecting all three classes of chemotactic receptors, or may alter the properties of neutrophil membranes or associated structures by a non-receptor-mediated mechanism which enhances chemotaxis independent of the specific stimulus. PF4(59–70) may thus represent an important probe for further studies of the extent to which chemotactic factor internalization and other cellular events modulate the functional activation of human neutrophils.

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REFERENCES

BOYUM A. (1968) Isolation of leukocytes from human blood: further observations. *Scand. J clin. Lab. Invest.* **21,** Suppl 97, 31.

BRINDLEY L.L., SWEET J.M. & GOETZL E.J. (1983) Stimulation of histamine release from human basophils by human platelet factor 4. *J. Clin. Invest.* **72,** 1218.

CHENOWETH D.E. & HUGLI T.E. (1978) Demonstration of specific C5a receptor on intact human polymorphonuclear leukocytes. *Proc. natn. Acad. Sci. U.S.A.* **75,** 3943.

DEUEL T.F., KEIM P.S., FARMER M. & HEINRIKSON R.L. (1977) Amino acid sequence of human platelet factor 4. *Proc natn. Acad. Sci. U.S.A.* **74,** 2256.

DEUEL T.F., SENIOR D., CHANG D., GRIFFIN G.L., HEINRIKSON R.L. & KAISER E.T. (1981) Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc. natn. Acad. Sci. U.S.A.* **78,** 4584.

DONABEDIAN H. & GALLIN J.I. (1981) Deactivation of human neutrophil chemotaxis by chemoattractants: effect on

receptors for the chemotactic factor f-met-leu-phe. *J. Immunol.* **127,** 839.

GOETZL E.J. (1980) A role for endogenous mono-hydroxy-eicosa-tetraenoic acids (HETEs) in the regulation of human neutrophil migration. *Immunology*, **40,** 709.

GOETZL E.J., BOEYNAEMS J.M., OATES J.A. & HUBBARD W.C. (1981) Stimulus-specificity of the chemotactic deactivation of human neutrophils by lipoxygenase products of arachidonic acid. *Prostaglandins*, **22,** 279.

GOETZL E.J., BRASH A.R., TAUBER A.I., OATES J.A. & HUBBARD W.C. (1980) Modulation of human neutrophil function by monohydroxy-eicosatetraenoic acids. *Immunology*, **39,** 491.

GOETZL E.J. & GORMAN R.R. (1978) Chemotactic and chemokinetic stimulation of human eosinophil and neutrophil polymorphonuclear leukocytes by 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT). *J. Immunol.* **120,** 526.

GOETZL E.J. & HOE K.Y. (1979) Chemotactic factor receptors of human PMN leukocytes. I. Effects on migration of labelling plasma membrane determinants with impermeant covalent reagents and inhibition of labelling by chemotactic factors. *Immunology*, **37,** 407.

GOETZL E.J. & PICKETT W.C. (1980) The human PMN leukocyte chemotactic activity of complex hydroxy-eicosatetraenoic acids (HETEs). *J. Immunol.* **125,** 1789.

GOLDMAN D.W. & GOETZL E.J. (1984) Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B_4. *J. exp. Med.* **159,** 1027.

GOLDMAN D.W., HANNAH A.L. & GOETZL E.J. (1982) Activation of human neutrophils by platelet factor 4. *Fed. Proc.* **41,** 272 (Abstract).

HANDIN R.I. & COHEN H.J. (1976) Purification and binding properties of human platelet factor four. *J. biol. Chem.* **251,** 4273.

LEABACK D.H. & WALKER P.G. (1961) Studies on glucosaminidase. IV. The fluorimetric assay of N-acetyl-beta-glucosaminidase. *Biochem. J.* **78,** 154.

NELSON R.D., MCCORMACK R.T., FIEGEL V.D. & SIMMONS R.L. (1978) Chemotactic deactivation of human neutrophils: evidence for nonspecific and specific components. *Infect. Immun.* **22,** 441.

OSTERMAN D.G., GRIFFIN G.L., SENIOR R.M., KAISER E.T. & DEUEL T.F. (1982) The carboxy-terminal tridecapeptide of platelet factor 4 is a potent chemotactic agent for monocytes. *Biochem. biophys. Res. Commun.* **107,** 130.

SCATCHARD G. (1949) The attraction of protein for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51,** 660.

STENDAHL O., COBLE, B.-I., DAHLGREN C., HED J. & MOLIN L. (1984) Myeloperoxidase modulates the phagocytic activity of polymorphonuclear neutrophil leukocytes. Studies with cells from a myeloperoxidase-deficient patient. *J. clin. Invest.* **73,** 366.

SULLIVAN S.J. & ZIGMOND S.H. (1980) Chemotactic peptide receptor modulation in polymorphonuclear leukocytes. *J. Cell Biol.* **85,** 703.

VITKAUSKAS G., SHOWELL H.J. & BECKER E.L. (1980) Specific binding of synthetic chemotactic peptides to rabbit peritoneal neutrophils: effects on dissociability of bound peptide, receptor activity and subsequent biological responsiveness (deactivation). *Molec. Immunol.* **17,** 171.

WARD P.A. & BECKER E.L. (1968) The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. *J. exp. Med.* **127,** 693.

ZIGMOND S.H. & HIRSCH J.G. (1973) Leukocyte locomotion and chemotaxis. New methods for evaluation of cell-derived chemotactic factor. *J. exp. Med.* **137,** 387.

ZIGMOND S.H., SULLIVAN S.J. & LAUFFENBURGER D.A. (1982) Kinetic analysis of chemotactic peptide receptor modulation. *J. Cell. biol.* **92,** 34.